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Role of oxygen in adaptive mutagenesis

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Abstract

Role of Oxygen in Adaptive Mutagenesis

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Spontaneous mutants are thought to occur primarily in dividing cells. However spontaneous mutations also arise in starving cells and in some cases this process appears to be adaptive. The well-characterized *Escherichia coli* strains FC29, FC40 and FC240 were used to measure and compare adaptive mutagenesis (Lac^- to Lac^+) under anaerobic and aerobic conditions. An anaerobic chamber was used, which allowed experiments to be performed in the absence of oxygen. It was found that the number of Lac^+ adaptive mutations was greatly decreased anaerobically compared to aerobic conditions. Addition of nitrate as an alternative electron acceptor in the selective medium did not increase the frequency of anaerobic mutagenesis. Several possibilities for the low level of anaerobic adaptive mutagenesis are available and include altered gene expression and the absence of mutagenic oxidative damage.

To my son, Akshay

Acknowledgements

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Table of Contents

List of Tables

List of Figures

Introduction

Adaptive mutagenesis implicates environmental stress factors as a cause of specific advantageous mutations and not just a selective force that enriches for random beneficial mutations within the population. The nonlethal genetic selection systems used to study adaptive mutation share basic tenets. In these systems beneficial mutations occur in the absence of significant cell growth and apparently without the detectable accumulation of non-advantageous mutations.

Starved populations of *Escherichia coli* can give rise to mutants even when the cells are not dividing or, apparently, replicating their genomes. This phenomenon has been called directed or adaptive mutation because in some cases only useful, not neutral or deleterious, mutations are thought to appear. Adaptive mutation has been well characterized in FC40, a strain of *E.coli* with a chromosomal deletion of the genes for lactose utilization and with a revertible *lac*$^-$ allele on an F´episome, F128. The episomal *lac* allele, *lacI33:: lacZ* , has a +1 frameshift mutation inactivating the *lacZ* gene. When FC40 is plated with lactose as its sole energy source, the cells do not proliferate, yet Lac$^+$ revertants appear at a constant rate for about a week. Furthermore, mutants do not arise even in the presence of lactose selection if the cells have some other, unfulfilled, growth requirement.

The purpose of this study is to test the possibility of a role for oxygen in adaptive mutagenesis by measuring Lac$^+$ reversion frequencies under aerobic and anaerobic conditions in genetically well-characterized *E.coli* strains.

Literature Review

A fundamental tenet of evolutionary biology is that mutations are random events. This tenet does not mean that mutation rates are unaffected by environmental factors or that all portions of the genome are equally susceptible to mutation. Indeed, enzymes that catalyze certain DNA repair processes are regulated by environmental factors, and many mutations are mediated by mobile elements that are not uniformly distributed in the genome (Campbell, 1983; Walker, 1984). This tenet of random mutation has recently been challenged (Cairns et al., 1988).

When populations of *Escherichia coli* are subjected to certain nonlethal selections, such as for utilization of a carbon source or reversion of an amino acid auxotrophy, spontaneous mutants accumulate with time, sometimes continuing to appear for weeks (Cairns et al., 1988). These unexpected findings suggested : (i) the mutants did not preexist but arose only after the cells were plated, (ii) the non-mutant cells were not growing, and (iii) the mutants would not have arisen had they not been selected for. These findings led Cairns et al. (1988) to suggest that " bacteria in stationary phase have some way of producing (or selectively retaining) only the most appropriate mutations". The phenomenon has variously been called "directed," "Cairnsian," "adaptive," and "selection- induced" mutation. Adaptive mutation will be the term used throughout this review although this only means that they arise after selection. In recent years, a growing number of studies have examined adaptive mutation in both bacteria and yeast. Adaptive mutation has provoked considerable controversy and experimentation (Cairns and Foster, 1991; 1992; Foster, 1993; Hall, 1992; Stahl, 1992) and is associated with recent challenges to the neo-Darwinian tenet that all mutations occur spontaneously without regard to fitness,

with natural selection increasing the frequency of advantageous alleles in a population.

Luria and Delbruck (1943) and Lederberg and Lederberg (1952) originally showed that mutations occur randomly prior to selection in a classic set of experiments. In these experiments the selective agent used, a bacteriophage or antibiotic, killed sensitive cells soon after exposure and it has been argued that only mutations that had occurred prior to exposure would be detected (Cairns et al.,1988). Sufficient time is not available after exposure for sensitive cells to mutate in response to the presence of the drug or phage.

Adaptive mutagenesis includes two major components: 1) cells can mutate while starving and not actively dividing and 2) only mutations that are beneficial or adaptive are produced under these conditions. The first part was initially demonstrated more than 40 years ago in a series of experiments conducted by Ryan and coworkers with *E.coli.* In the first set of experiments, several hundred tubes containing minimal medium supplemented by a small amount of histidine were inoculated with a small number of His^- cells and then incubated for two days. A small amount of growth occurred in most tubes because of the histidine but some cultures were saturated because a His^+ reversion event had occurred during cell division (Ryan, 1955). The cultures were left to incubate for several weeks and additional tubes became saturated over this peroid of time. Ryan (1955) concluded that in these latter cultures a His^- to His^+ reversion event had occurred in cells that were starving and no longer actively dividing. Later, Ryan and coworkers used a heavy isotope of nitrogen to show that no detectable DNA replication was occurring in such cultures (Ryan et al., 1961).

The results of Ryan's experiments that indicated mutations can occur in starving, non-dividing cells were largely ignored until after the publication of the work of Cairns et al. (1988). Cairns et al. (1988) demonstrated that Lac^- to Lac^+ reversion events occur on a selective medium with lactose as the sole carbon source over a period of time. The distribution of Lac^+ revertants did not follow the so-called Luria-Delbruck distribution expected if the reversions were random events occurring prior to selection but instead conformed to a Poisson distribution that would be expected of mutational events occurring after selection.

In contrast, mutations to valine-resistance did not accumulate over time on the lactose selective plates. These results were interpreted to mean that cells are preferentially able to produce mutational events that result in useful phenotypes and this has become the most controversial aspect of adaptive mutagenesis.

Several authors have attributed the Poisson distribution obtained by Cairns et al. (1988) to experimental artifacts (Dijkmans et al., 1994; Lenski et al., 1989; Lenski and Mittler., 1993; Stewart et al., 1990). Nonetheless subsequent studies have reported the occurrence of adaptive mutations in yeast (Steele and Jinks-Robertson., 1992) as well as additional examples in *E.coli* (Cairns and Foster., 1991; Hall., 1988,1990, 1991, 1992). So far no definitive experiments have been reported that clearly demonstrate the presence or absence of a mechanism that can preferentially generate beneficial mutations. Most recent experiments in adaptive mutagenesis have focused on elucidating at the molecular level how mutations are produced by starving, non-dividing cells regardless of whether or not only beneficial events are created.

Most of these recent experiments in *E.coli* have utilized the FC29 and FC40 strains, which carry chromosomal deletions of the *lac* region, to probe the mechanisms of adaptive mutagenesis (Cairns and Foster, 1991). FC40 is Rif^R and carries a F′ with a *lacI lacZ* fusion that eliminates the coding sequence for the last four residues of *lacI* , all of *lacP* and *lacO* and the first 23 residues of *lacZ* (Cairns and Foster, 1991). Transcription is started constitutively from the $lacI^q$ promoter but the strain is Lac^- because of a polar +1 frameshift mutation changing CCC to CCCC at the 320 th codon of *lacI* . It can revert to Lac^+. FC29 is Rif^S and carries a F′ Δ (*lacI lacZ*) . This strain cannot revert to Lac^+.

Experiments are usually performed by spreading both strains on lactose-selective plates. The FC29 cells act as scavengers to consume any contaminating glucose that may be present (Cairns and Foster, 1992). After both strains are grown to saturation and plated they are incubated for several days and Lac^+ revertants scored each day. Revertants that appear after one or two days are assumed to be random and to have occurred prior to selection while Lac^+ colonies that appear later are adaptive and have occurred during selection.

Rosenberg et al. (1994) and Foster and Trimarchi (1994) have shown that these adaptive Lac^+ mutations of FC40 are primarily one-base deletions in a run of repeated bases. In contrast, spontaneous or random mutations that occur prior to selection are a more diverse group of DNA changes and include duplications and larger deletions as well as the one-base deletions (Foster and Trimarchi ,1994). These data suggest that the molecular mechanisms of random and adaptive mutagenesis may be different. Harris et al. (1994) have constructed *recA*$^-$ and *recB*$^-$ derivative FC40 strains and found that adaptive mutagenesis does not occur in either derivative, although random, spontaneous

mutations still occur. Since the *recA* and *recB* genes are involved in homologous recombination (Clark and Low, 1988), Harris et al. (1994) suggest that recombination is required for adaptive mutagenesis. In support of this hypothesis, they showed that a null *recD* allele which is hyper-recombinagenic (Rosenberg and Hastings, 1992) also results in hypermutability (Harris et al., 1994).

Radicella et al. (1995) have recently shown that the *lacI33* allele that reverts to Lac^+ in the FC40 strain must be located on the F´plasmid in order for adaptive mutagenesis to occur. When the *lacI33* allele was moved to its normal chromosomal location, adaptive mutagenesis was reduced 25 - to 50 - fold (Radicella et al .,1995).

Galitski and Roth (1995) have further shown that adaptive mutagenesis depends on the transfer functions of the F factor. The F plasmid controls its sexual functions via a fertility inhibition (Fin) system in which the products of two genes, *fin O* and *fin P*, interact to form a Fin OP inhibitor of the expression of the transfer (*tra*) genes involved in conjugation (Lee et al ., 1992). The *fin O* gene of the sex factor is inactivated by the presence of an IS3 insertion which results in a high level of constitutive *tra* gene expression (Cheah and Skurray, 1986). Galitski and Roth (1995) repressed *tra* gene expression and conjugal activity of the F´ plasmid of FC40 by supplying Fin O activity via a second plasmid. This resulted in the elimination of adaptive mutagenesis. The authors suggested that adaptive mutations may arise during DNA replication of the F´ beginning at the transfer origin.

Foster and Trimarchi (1995) demonstrated that *tra* mutations which strongly inhibit conjugation of the F´ plasmid only produced a modest decrease in the number of adaptive mutations. Typically in adaptive mutagenesis

experiments about 10^8 FC 40 cells are plated on selective lactose plates with a 10-fold excess of FC29 Lac$^-$ scavenger cells (Cairns and Foster, 1991). The scavengers carry a F´with part of the *lacZ* gene deleted so they cannot revert to Lac$^+$. Normally conjugation and transfer of the F plasmid does not occur between F-containing cells but F transfer has been shown to occur between starved male cells (Peters and Bension , 1995).

Therefore Foster and Trimarchi tested to see if the Lac$^+$ adaptive mutations arose through conjugation of the F´ from donor FC40 cells to recipient FC29 cells. The vast majority of Lac$^+$ mutations were FC40 cells indicating that adaptive mutagenesis was occurring in the absence of actual conjugation. Foster and Trimarchi (1995) concluded that adaptive mutagenesis requires conjugal functions but not actual conjugation.

Lac$^+$ adaptive mutagenesis appears to be the result of DNA polymerase errors during DNA synthesis. *E.coli* contains three DNA polymerases. DNA polymerase III is a complex of at least 10 subunits and has a major role in DNA replication (Mc Henry, 1988). DNA polymerase I consists of a single polypeptide and has a role in DNA synthesis during DNA repair and replacing RNA primers during chromosomal replication (Kornberg and Baker, 1992). Although a discrete role for DNA polymerase II has been long sought, none has been unambiguously established. The structural gene, *polB* , for DNA polymerase II has been cloned and shown to be regulated as part of the SOS response to DNA damage (Bonner et al., 1990; Chen et al., 1990; Iwasaki et al., 1990).

It has been recently shown that a FC40 *polB* deletion (*polBΔ1*) strain had an increased sensitivity to killing by hydrogen peroxide (H_2O_2) and displayed a threefold increase in adaptive mutagenesis (Escarceller et al., 1994). This suggested that DNA polymerase II is active in non-dividing cells but

another, less accurate DNA polymerase, can substitute for it. In a second study the FC40 chromosomal *polB*$^+$ gene was replaced by an exonuclease-deficient mutant DNA polymerase II allele (*polBexI*). The *polBexI* allele increased adaptive Lac$^+$ mutagenesis 4- to 6-fold (Foster et al., 1995).

DNA polymerase III is encoded by the *dnaE* gene (Gefter et al., 1971). The addition of an antimutator allele of *dnaE*,*dnaE 915* (Fijalkowska and Schaaper, 1993), to the *polBΔ1* strain reduced Lac$^+$ adaptive mutagenesis to the level of *polB* $^+$*dnaE915* cells. Foster et al. (1995) concluded that the majority of Lac$^+$ adaptive mutations occur by uncorrected errors made by DNA polymerase III during conjugal DNA synthesis.

Why DNA polymerase III activity during F plasmid synthesis is so error-prone is not clear. One possibility is oxidative DNA damage that leads to polymerase mistakes. DNA polymerase II, which is active in non-dividing cells as described above, is part of the SOS regulon (Bonner et al, 1990) and is capable of insertion and bypass at abasic sites (Bonner et al.,1988; Tessman and Kennedy.,1993). DNA polymerase III is also active in non-dividing cells and apparently makes more errors than DNA polymerase II. If abasic sites or any other premutagenic lesion results from oxidative damage, failure to replicate past these sites accurately by DNA polymerase III (or II) may result in Lac$^+$ adaptive mutations. So far there is no report of the effect of oxygen on adaptive mutagenesis. The experiments to be described were planned and performed to determine if oxygen does have a role in adaptive mutagenesis.

Materials and Methods

Bacterial Strains

The strains used , FC29, FC40 and FC240, were obtained from Dr. Pat Foster (Department of Environment Health, Boston University School of Medicine). All the strains are derivatives of P90C (Coulondre and Miller, 1977) , and were constructed using standard methods (Miller, 1972). FC40 and the scavenger strain, FC29, have been previously described (Cairns and Foster,1991). Briefly, FC40 is rifampicin-resistant (Rif^R) and has an F´carrying a *lacI-lacZ* fusion with a +1 base pair frameshift mutation, *lacI33* , in the *lacI* coding sequence that inactivates the *lacZ* gene. Phenotypically this strain is Lac^- but reverts to Lac^+ by a variety of events (Calos and Miller ,1981). FC29 is rifampicin-sensitive (Rif^S) and has an F´carrying a deletion allele of *lacZ* . FC240 is the same as FC40 but it has the *recA430* allele instead of $recA^+$ .This strain can revert to Lac^+ but it lowers the rate of adaptive mutagenesis (Cairns and Foster, 1991). All three strains are deleted for the chromosomal *lac* operon.

Media

Selective M9 minimal plates were prepared as follows: 3g of Na_2PO_4, 1.5g of KH_2PO_4, 0.25g of NaCl, 0.5g of NH_4Cl were added to one litre of distilled H_2O. After autoclaving, 10ml of a 0.01M solution of $CaCl_2$, 1ml of a 1M solution of $MgSO_4 \cdot 7H_2O$, 10ml of a 20% solution of lactose and 2ml of B1 vitamin were added and solidified with 1.5% agar (Miller ,1972 ; Cairns and Foster ,1991).The frequency of Lac^+ revertants was also determined on M9 plates containing 0.2 % lactose and 0.1% KNO_3 solidified with 1.5% agar. Tryptone plates consisted of 1% tryptone and 0.5% NaCl solidified with 1.5% agar. Bacteria were cultivated in liquid M9 minimal medium supplemented with

0.2% glucose. Sterile saline (0.85%) was utilized for washing and diluting the cultures.

Sterilization

Glucose, agar, salts, tryptone and NaCl were autoclaved before use at standard pressure and temperature. Lactose was filter-sterilized using a 0.22 micron filter and stored in sterile jars.

Mutagenesis Procedures

Lac^+ reversion frequencies were measured by growing duplicate or triplicate inoculates of all the strains to saturation in M9 minimal medium at 37^oC. The cultures were then centrifuged, resuspended in saline or M9 medium, and plated on M9 minimal medium with lactose to select for Lac^+ revertants and on tryptone to determine cell titers at saturation. All the manipulations of the anaerobic cultures were performed in the anaerobic chamber (except washing by centrifugation) while the aerobic cultures were manipulated on the laboratory bench. A total of 10^8 FC40 or FC240 cells were added to each plate, a number previously shown to be satisfactory (Cairns and Foster, 1991). About 10^9 cells of FC29, a Lac^- scavenger, were also plated to remove any contaminating glucose. The plates were incubated at 37^oC for 48 hours to determine saturation titers, for 7-8 days to select for Lac^+ revertants on the laboratory bench and for up to 15 days in the anaerobic chamber. Some experiments were done without using FC29.

The following procedures were done to test whether Lac^+ revertants recovered aerobically can grow in the anaerobic chamber on the selective medium. Selected Lac^+ revertants of FC40 on M9-lactose minimal plates were purified and grown to saturation in the chamber. They were then diluted and

plated on M9-lactose minimal plates to see if the revertants form colonies under anaerobic conditions.

Anaerobic Chamber

The anaerobic chamber (Anaerobe Systems, San Jose, CA) consists of an air-tight acrylic chamber 152.4cm L x 76cm W x 44.7cm H. The armport system permits access to the interior of the chamber which consists of three parts: a. Sleeve assembly (a heavy duty rubber sleeve, a plastic cuff ring and a soft rubber arm cuff responsible for making a snug seal around the arm while working in the chamber without gloves); b. Armport door (used to seal the armports while the chamber is in active use); and c. Foot pedals (two foot operated valves, one for gas and the other is for vacuum, used to make the sleeve area anaerobic before removing the armport doors to insert the arms into the chamber). A transfer module for moving materials in and out of the chamber and an incubator kept at $37^{o}C$ are attached to the side of the chamber. The catalyst basket consists of aluminium pellets coated with palladium which are present to remove trace amounts of oxygen. The catalyst is reactivated every three days by heating at $160^{o}C$ for 2 hours. A positive pressure of ca.7.6 cm of water is provided by an electronic pressure controller to maintain the gaseous environment of the chamber. Anaerobic conditions are established initially in the chamber by use of a displacement balloon to physically expel air from the chamber. Then the chamber is refilled with a gas mixture of 10% hydrogen (H_2), 10% carbon dioxide (CO_2) and 80% nitrogen (Liquid Carbonic Corp., San Carlos, CA.). This procedure is repeated twice to ensure the chamber is anaerobic.

Results

The production of Lac^+ revertants was followed for strains FC40 (*recA*$^+$) and FC240 (*recA430*) in an aerobic environment (Figure 1). As expected for FC40, after two days Lac^+ revertants increased sharply in a linear fashion until seven days when the experiment was terminated. At this point each selective plate contained several hundred Lac^+ colonies. These experiments were done either using the FC40 strain alone or plating both FC40 and the scavenger strain FC29. The production of Lac^+ revertants was similar in both cases although more revertants occurred on plates spread with FC40 alone (Figure 1).

The FC240 strain displayed a much smaller increase in Lac^+ revertants after day two (Figure 1). By the end of the experiment at seven days usually fewer than 100 Lac^+ revertants had accumulated on the selective plates. There was a small increase in the number of accumulated Lac^+ revertants when both FC240 and FC29 were plated compared with FC240 alone.

In contrast to the aerobic results, Lac^+ revertants accumulated very slowly with FC40 in the anaerobic chamber (Figure 2). At the end of 15 days most selective plates contained 20 or fewer Lac^+ colonies and there was little difference in values between experiments with FC40 and FC29 and FC40 alone. Lac^+ revertants also accumulated slowly with FC240 and averaged fewer than 10 per plate after 15 days (Figure 2).

Experiments were done to determine if the addition of an alternative electron acceptor, nitrate, in the form of potassium nitrate (KNO_3) to the selective medium, would have any effect on the frequency of Lac^+ revertants. It might be possible that the presence of nitrate would increase cellular energy production anaerobically and allow more adaptive mutagenesis to occur.

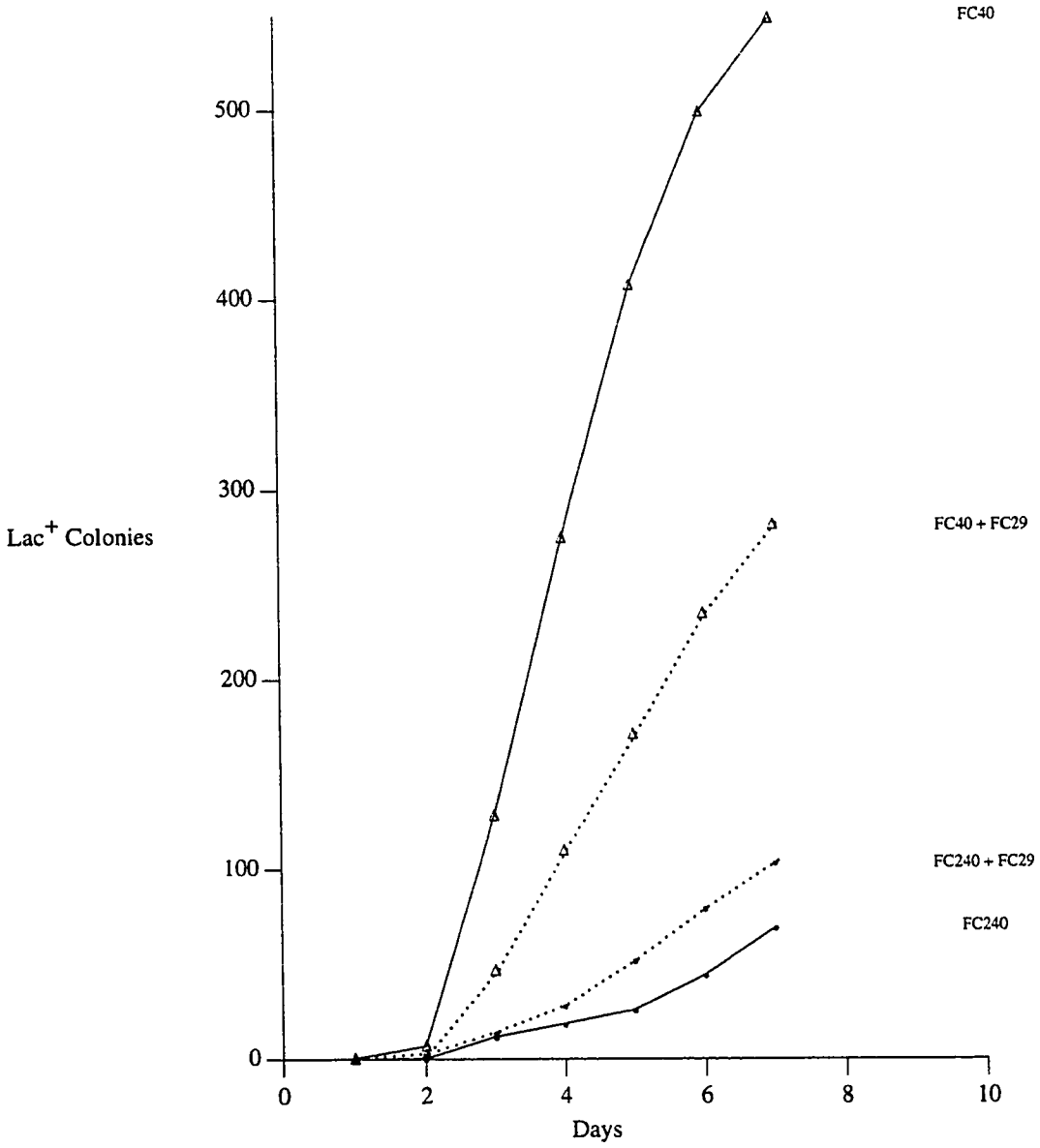

Fig. 1. The aerobic accumulation of Lac^+ revertant colonies of FC40, FC240, FC40+FC29 and FC240+FC29 on M9-lactose plates. Cultures of each strain were grown to saturation in M9-minimal medium. Aliquots of 0.075ml, containing 10^8 FC40 cells, were mixed with 0.025 ml containing 10^9 FC29 scavenger cells and spread on M9-lactose plates. The number of Lac^+ revertant colonies on each plate was counted each day. The Lac^+ colony counts are the average for 11, 4, 16, and 9 plates respectively.

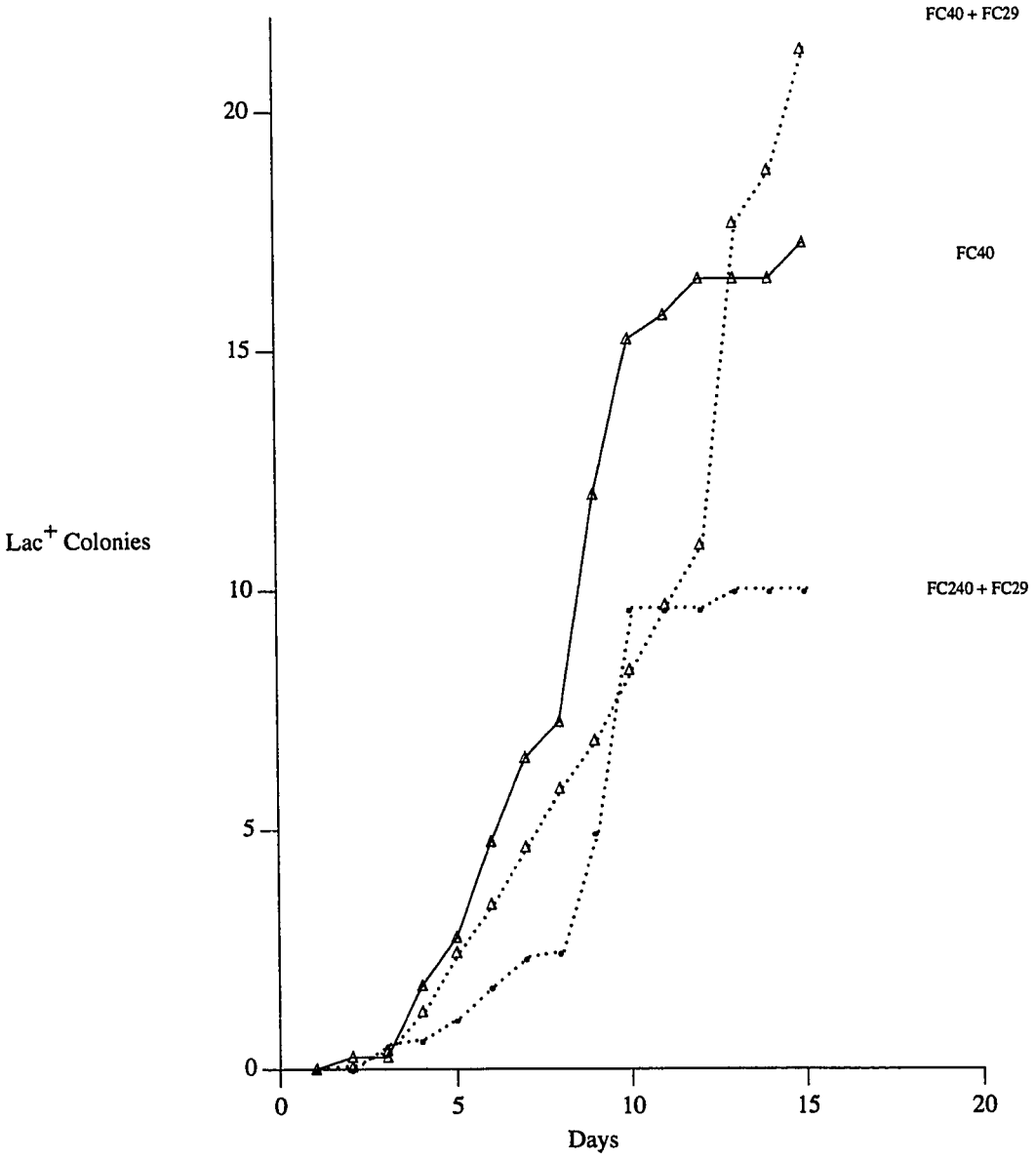

Fig. 2. The anaerobic accumulation of Lac^+ revertant colonies of FC40, FC40+FC29 and FC240+FC29 on M9-lactose plates. Cultures of each strain were grown to saturation in M9-minimal medium. Aliquots of 0.075ml, containing 10^8 FC40 cells, were mixed with 0.025 ml containing 10^9 FC29 scavenger cells and spread on M9-lactose plates. The number of Lac^+ revertant colonies on each plate was counted each day. The Lac^+ colony counts are the average for 4, 16, and 7 plates respectively.

However, as is shown in Tables 1 and 2, the addition of KNO_3 to the selective medium did not alter the frequency of Lac^+ revertant accumulation either aerobically (Table 1) or anaerobically (Table 2).

The low numbers of Lac^+ revertants that accumulate anaerobically (Figure 2) may be due to the inability of many of the plated cells to revert to Lac^+ after a few days in the anaerobic chamber. To test this possibility, FC40 cultures spread on selective plates were initiated anaerobically. Some culture plates were removed from the anaerobic chamber after five or 10 days of incubation and then further incubated aerobically for up to 15 days. The remaining cultures stayed in the chamber and continuously incubated anaerobically for 15 days. As shown in Figure 3, after two or three days of aerobic incubation the cultures removed from the anaerobic chamber after five or 10 days begin to display the sharp linear increase in Lac^+ revertants that is characteristic of FC40 cultures plated aerobically (Figure 1). Clearly FC40 cells still maintain the ability to revert to Lac^+ after incubating anaerobically for 10 or more days.

It is possible that FC40 cells may continuously revert to Lac^+ at a high frequency anaerobically but many or most of these Lac^+ cells are unable to form visible colonies in the anaerobic chamber. To test this possibility, purified Lac^+ revertants were selected which had appeared during aerobic experiments (Figure 1). These Lac^+ revertants were used to initiate anaerobic cultures that were grown to saturation in the chamber. The cultures were diluted and spread on selective plates. Some plates remained in the chamber to incubate and the others were removed from the chamber after spreading and incubated aerobically. Lac^+ colonies were counted on all plates when they first appeared.

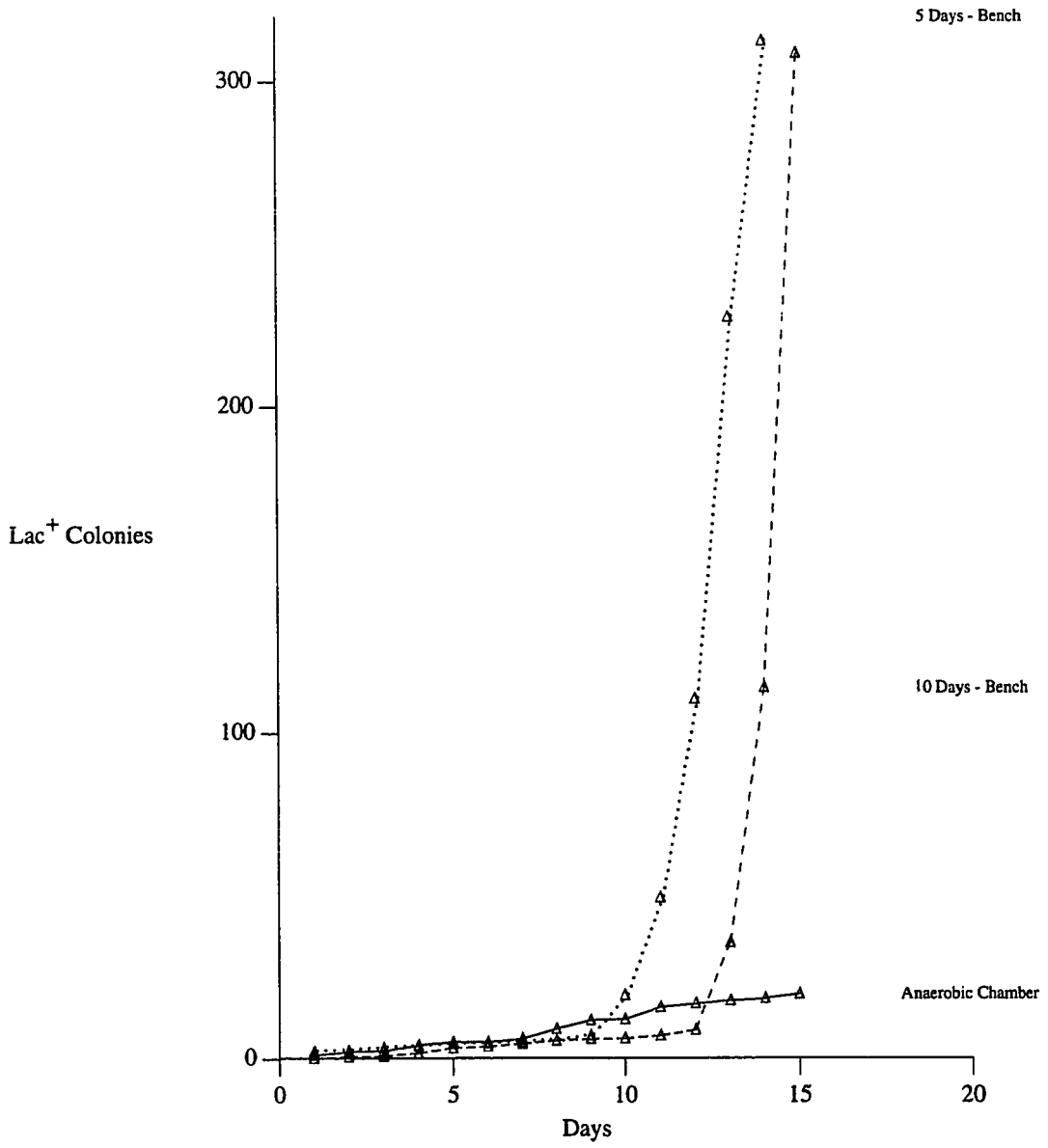

Fig. 3. The accumulation of Lac^+ revertant colonies of FC40+FC29 on M9-lactose plates. Cultures of FC40 and FC29 were grown to saturation in M9-minimal medium. Aliquots of 0.075ml, containing 10^8 FC40 cells, were mixed with 0.025 ml containing 10^9 FC29 scavenger cells and spread on M9-lactose plates. The number of Lac^+ revertant colonies on each plate was counted each day. Some plates were transferred from the anaerobic chamber to an aerobic incubator (bench) after 5 days. Other plates were transferred after 10 days and the rest remained in the chamber.

Table1

Effect of KNO_3 on Lac^+ revertant accumulation in an aerobic environment[a]

	Lac^+ colony count	
Day	M9-lactose plates	M9-lactose+KNO3 plates
1	0	0
2	3	4
3	20.33	24.66
4	51.66	58.66
5	87.66	80.00
6	125.66	102.00
7	169.00	118.66

[a] Lac^+ revertants of FC29+FC40 after plating on M9-lactose plates and M9-lactose+KNO_3 plates. The number of Lac^+ colonies on each plate was counted each day. The Lac^+ colony counts are the average for three plates.

Table 2

Effect of KNO_3 on Lac^+ revertant accumulation in an anaerobic environment[a]

Day	Anaerobic Lac^+ count	
	M9-lactose plates	M9-lactose+KNO3 plates
1	3	0
2	7	5.6
3	10.5	6.3
4	11	6.3
5	11	6.3
6	11	6.3
7	13	7.6
8	13	7.6
9	13	8.3

[a] Lac^+ revertants of FC29+FC40 after plating on M9-lactose plates and M9-lactose+KNO_3 plates. The number of Lac^+ colonies on each plate was counted each day. The Lac^+ colony counts are the average for two plates.

As shown in Table 3, most of the colonies that were counted for the aerobic cultures first appeared after one day of incubation. The same Lac^+ revertants incubated anaerobically had some Lac^+ colonies first appear during each of the four days of the experiment. In addition the total number of Lac^+ colonies that appeared for the anaerobic cultures were only about 20% of the total that appeared for the same Lac^+ revertants incubated aerobically (Table 3). Apparently the anaerobic plating efficiency for at least some Lac^+ cells is significantly less than the aerobic plating efficiency and may account for some part of the large difference between the aerobic and anaerobic Lac^+ reversion frequencies shown in Figures 1 and 2.

Purified Lac^+ revertants were also selected that had originally appeared during anaerobic experiments (Figure 2). These Lac^+ revertants were analyzed in experiments identical to those described above and the results are shown in Table 4. All of the Lac^+ colonies that were counted for the aerobic experiments first appeared after one day of incubation while most of the Lac^+ colonies that were counted for the anaerobic experiments first appeared after two days of incubation. In these experiments the total number of Lac^+ colonies for the aerobic and anaerobic Lac^+ revertant cultures were similar.

Table 3

Colony formation of Lac+ revertants that were originally selected aerobically[a]

Lac+ Revertants	Aerobic colony count Colonies first appeared				Anaerobic colony count Colonies first appeared			
	Day1	Day2	Day3	Day4	Day1	Day2	Day3	Day4
D_2[b]FC40+FC29	18	2	0	0	1	0	0	3
D_3FC40+FC29	18	0	1	0	1	1	1	1
D_6FC40+FC29	14	0	0	0	2	0	0	2

[a] Lac+ revertants were grown to saturation anaerobically, diluted and plated anaerobically on M9-lactose plates and subsequently incubated both aerobically and anaerobically.

[b] Lac+ revertant colonies of days 2, 3 and 6 were selected.

Table4

Colony formation of Lac+ revertants that were originally selected anaerobically[a]

Lac+ Revertants	Aerobic colony count Colonies first appeared				Anaerobic colony count Colonies first appeared			
	Day1	Day2	Day3	Day4	Day1	Day2	Day3	Day4
D_5[b]FC40+FC29	16	0	0	0	0	41	3	0
D_7FC40+FC29	45	0	0	0	0	28	4	0
D_{10}FC40+FC29	41	0	0	0	0	19	2	4

[a] Lac+ revertants were grown to saturation anaerobically, diluted and plated anaerobically on M9 -lactose plates and subsequently incubated both aerobically and anaerobically.

[b] Lac+ revertant colonies of days 5, 7 and 10 were selected.

Discussion

The experiments of this study have characterized the frequency of adaptive mutations to Lac^+ in the presence and absence of oxygen using the well - characterized FC40 and FC240 strains. Aerobically the results are similar to those previously obtained by Cairns and Foster (1991, 1992). With FC40 (*recA*$^+$) , large numbers of Lac^+ colonies begin to appear at about two days of incubation and there is a sharp linear increase in Lac^+ frequencies for the next few days. In the case of FC240 (*recA430*) , Lac^+ colonies appear at about two days of incubation but there is only a gradual increase in Lac^+ frequencies for the remainder of the experiments. The *recA430* allele is defective in the cleavage of the LexA and UmuD proteins (Ennis et al., 1985; Nohmi et al., 1988; Shinagawa et al., 1988). However it is not likely that the *umuC* or *umuD* genes are involved in adaptive mutagenesis since the Lac^+ reversion frequency was not affected in a strain carrying a *umuC*$^-$ allele (Cairns and Foster, 1991). Moreover the low level of Lac^+ reversion with *recA430* is probably not completely due to the inability to cleave LexA normally since a *lexA3* strain, which produces a LexA repressor that is resistant to cleavage by RecA (Little et al., 1980) , shows a small decrease in Lac^+ reversion compared to that shown by *recA430* (Cairns and Foster, 1991). The additional RecA function that is impaired with the *recA430* allele and is needed for a full level of Lac^+ mutagenesis is not known.

In the anaerobic chamber, the level of Lac^+ adaptive mutagenesis was very low although a small amount appeared to occur. Although the FC40 strain showed higher levels of mutagenesis than FC240, the increase was much less than was demonstrated aerobically. A number of possibilities must be considered to explain the low level of anaerobic Lac^+ mutagenesis. It may be

argued that Lac$^-$ cells die on the selective plates in the anaerobic chamber before they have the opportunity to mutate to Lac$^+$ and form visible colonies. This possibility is eliminated by experiments where FC40 cultures were grown and spread on selective plates anaerobically. Some of the plates were removed from the anaerobic chamber after five days and then incubated aerobically, some plates were removed and incubated aerobically after 10 days and the remaining plates remained in the chamber. After removal from the chamber, the plates incubated aerobically showed the characteristic appearance of Lac$^+$ revertants which accumulated in large numbers for several days. Even after 10 days on selective plates in the anaerobic chamber, the Lac$^-$ cells retained the ability to mutate to Lac$^+$ when incubated aerobically.

Possibly anaerobic cells have an insufficient level of energy production to carry out high levels of a mutagenic process. The M9-lactose selective medium used in these experiments lacks an obvious alternative electron acceptor, such as nitrate, to engage in anaerobic respiration. Presumably the only energy source for these cells is endogenous substrates that can be used for fermentation. Nitrate (NO_3^-) was added to the selective medium as potassium nitrate, but the production of Lac$^+$ revertants was unchanged both anaerobically and aerobically.

It is also possible that Lac$^+$ mutations do occur anaerobically at or near aerobic frequencies but most of these revertant cells are unable to form visible colonies in the chamber. This possibility was tested by growing Lac$^+$ revertants that were originally isolated as adaptive mutations aerobically to saturation anaerobically. The cultures were then diluted appropriately so that 100 or fewer cells could be spread on M9-lactose selective plates. One half of the plates were incubated anaerobically and the remaining plates were incubated

aerobically; the resulting colony titers were compared. The colony forming ability of these Lac^+ revertants did appear to be lowered by the anaerobic environment with colony titers about 20% of the aerobic levels. The experimental conditions here are not identical to the aerobic and anaerobic experiments done to determine Lac^+ adaptive mutagenesis frequencies. In the latter case, millions of Lac^- cells are spread on selective plates and the few Lac^+ revertants that form visible colonies are observed while in the former case, 100 or fewer Lac^+ cells are spread on selective plates to observe how many will form visible colonies. Nonetheless, it seems reasonable to conclude that possibly some of the large difference between aerobic and anaerobic Lac^+ mutation frequencies may be due to the inability of some Lac^+ revertants to form visible colonies anaerobically.

Assuming that not all of the difference between aerobic and anaerobic Lac^+ reversion frequencies can be accounted by the inability of some Lac^+ cells to form visible colonies, other possibilities can be considered. The presence or absence of oxygen may affect the expression of genes whose products are involved in the pathway(s) of adaptive mutagenesis. The *lacI33::lacZ* mutation of FC40 is carried on a sex factor and it has been shown that F plasmid gene (*tra*) expression is essential for adaptive mutagenesis (Foster and Trimarchi, 1995; Galitski and Roth, 1995). If an anaerobic environment results in reduced *tra* gene expression, lower levels of adaptive mutagenesis would be expected. However, it has been found that anaerobic conditions actually result in an increase in *tra* gene expression (Silverman et al., 1991). This does not eliminate the possibility that an anaerobic environment may still alter expression of some other F plasmid or chromosomal genes that could affect the production of adaptive mutations.

Finally there is the possibility that at least part of adaptive mutagenesis is due to oxidative damage to DNA. Oxidative damage to DNA (reviewed in Friedberg et al., 1995) involves attack by reactive oxygen species and leads to a variety of radical-induced DNA lesions. Some of these lesions may result in mutations after DNA synthesis. DNA polymerase II is the one DNA polymerase of the three possessed by *E.coli* for which a defined role has not yet been established. DNA polymerase II, which is encoded by the *polB* gene, is a part of the SOS regulon and has been shown to synthesize past an abasic lesion *in vitro* and *in vivo* (Bonner et al., 1990; Iwasaki et al., 1990; Tessman and Kennedy, 1993; Bonner et al., 1988). Cells with a deleted *polB* gene demonstrated a 5- to 10-fold increase in killing by hydrogen peroxide (H_2O_2), which causes oxidative damage, compared with *polB*$^+$ cells (Escarceller et al., 1994). The same study also showed that the frequency of Lac$^+$ adaptive mutations was also increased about 3-fold in the deleted *polB* strain.

A more recent study showed that a strain with an exonuclease-deficient (or proofreading-deficient) pol II mutation (*polBex1*) had a higher frequency of Lac$^+$ adaptive mutagenesis than an isogenic *polB*$^+$ strain (Foster et al.,1995). The presence of the antimutator allele of *dnaE* , *dnaE915* (Fijalkowska and Schaaper, 1993), decreased adaptive mutagenesis by one-third in both *polB*$^+$ and *polB* deletion strains (Foster et al.,1995). Since *dnaE* is the structural gene for the alpha subunit of DNA polymerase III (McHenry, 1988), it was concluded that DNA synthesis by DNA polymerase III is responsible for much of Lac$^+$ adaptive mutagenesis. When a wild-type DNA polymerase II is present with proofreading capacity, it apparently is able to synthesize F plasmid DNA with a relatively high fidelity, whereas synthesis with DNA polymerase III is more error-prone (Foster et al.,1995).

It may be suggested that oxidatively damaged F plasmid DNA is a template for synthesis by both DNA polymerase II and III. The induced lesions preferentially result in mutations during synthesis by DNA polymerase III compared with DNA polymerase II. In the absence of oxidative damage or similar lesions, synthesis results in few mutations, regardless of which DNA polymerase is used. Further experiments are needed to distinguish among the possible explanations, which are not mutually exclusive, for the anaerobic reduction in adaptive Lac^+ mutagenesis.

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